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AAL-Toxin, A Potent Natural Herbicide which Disrupts Sphingolipid Metabolism of Plants*

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Abstract: AAL-toxin, a product of *Alternaria alternata* (Fr.) Keissl., is effective as a herbicide at low concentrations against a range of broadleaf plants (e.g. jimsonweed, prickly sida and black nightshade). However, monocotyledonous crops such as maize and wheat, as well as some varieties of tomato, are tolerant to it. The IC_{50} values for cellular electrolyte leakage and chlorophyll loss in duckweed (*Lemna pausicostata* L.) after 72 h treatment were 20–40 nM. Similar results were obtained with a susceptible tomato variety. AAL-toxin caused rapid cellular leakage of electrolytes, followed by cellular collapse; the first symptom at the ultrastructural level is disruption of the plasma membrane. The effects of the toxin are not light-dependent and appear to be associated with dysfunction of the plasma membrane. Fumonisins and sphingoid bases such as phytosphingosine cause similar effects, although these compounds are less potent (fumonisins, about 10-fold; sphingoid bases, about 100-fold). Recent studies suggest that in duckweed and in susceptible tomato varieties, AAL-toxin- and fumonisin B_1-induced disruption of sphingolipid metabolism is an early event in the cascade of events leading to phytotoxic injury and cell death.

Key words: AAL-toxin, *Alternaria alternata* (Fr.) Keissel., sphingolipid metabolism, herbicide.

1 INTRODUCTION

The genus *Alternaria* is widely distributed in nature and a number of *Alternaria* spp are pathogenic to plants, examples being *A. crassa* (Sacc.) Rands on jimsonweed[1] (*Datura stramonium* L.); *A. cassiae* on sicklepod[2] (*Cassia obtusifolia* L.); *A. macrospora* Zimmerman on spurred anoda[3] (*Anoda cristata* (L.) Schlecht), and *A. alternata* (Fr.: Fr.) Keissl. f. sp. *tenuis* on morning glory[4] (*Ipomoea purpurea* (L.) Roth). Many species of *Alternaria* produce phytotoxins that may be related to their pathogenicity.[5–9] AAL-toxin was first isolated from *A. alternata* f. sp. *lycopersici* in 1981 by Bottini *et al.*[10] It was determined to be the active factor in stem canker disease in susceptible tomatoes (*Lycopersicon esculentum* Mill).[8,11–15] Susceptible tomatoes carry the recessive genotype (*asc/asc*); resistant (*Asc/Asc*) and heterozygous (*Asc/asc*) tomatoes are minimally affected by *A. alternata* or AAL-toxin.[5,6,11,16,17]

Initial reports suggested that AAL-toxin was host-specific to susceptible tomatoes, as was *A. alternata* f. sp. *lycopersici*.[8,12–15] However, because fumonisin B_1, a

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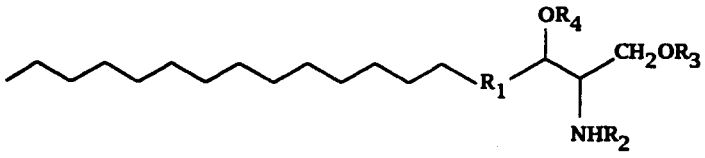
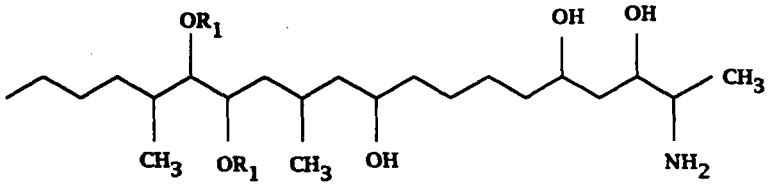
SPHINGOLIPIDS



Sphingolipid	R_1	R_2	R_3	R_4
Sphingosine	CH=CH	H	H	H
Phytosphingosine	CH_2-CHOH	H	H	H
Sphinganine	CH_2-CH_2	H	H	H
Tetraacetyl-phytosphingosine	CH_2-CH(O-CO-CH_3)	CO-CH_3	CO-CH_3	CO-CH_3
N-Lignoceroyl-D,L-sphinganine	CH_2-CH_2	CO-$(CH_2)_{22}$-CH_3	H	H

FUMONISIN



Toxin	R_1	R_2	R_3	R_4	R_5
FB_1	CO-CH_2-CH(CO_2H)-CH_2CO_2H				
TA_1	H	CO-CH_2-CH(CO_2H)-CH_2CO_2H	OH	OH	H
TA_2	CO-CH_2-CH(CO_2H)-CH_2CO_2H	H	OH	OH	H
TB_1	H	CO-CH_2-CH(CO_2H)-CH_2CO_2H	H	OH	H
TB_2	CO-CH_2-CH(CO_2H)-CH_2CO_2H	H	H	OH	H
TC_1	H	CO-CH_2-CH(CO_2H)-CH_2CO_2H	H	H	H
TC_2	CO-CH_2-CH(CO_2H)-CH_2CO_2H	H	H	H	H
TD_1	H	CO-CH_2-CH(CO_2H)-CH_2CO_2H	H	OH	C(=O)CH_3
TD_2	CO-CH_2-CH(CO_2H)-CH_2CO_2H	H	H	OH	C(=O)CH_3
TE_1	H	CO-CH_2-CH(CO_2H)-CH_2CO_2H	H	H	C(=O)CH_3
TE_2	CO-CH_2-CH(CO_2H)-CH_2CO_2H	H	H	H	C(=O)CH_3

AAL-TOXINS

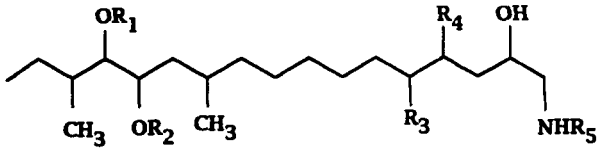


Fig. 1. Chemical structures of compounds mentioned in the text.

structurally related phytotoxin,[18] was known to have a broader host range,[19–27] AAL-toxin was tested on a wide range of plant species and was found to be phytotoxic to a number of weed and crop species.[7,17,26–30]

Because various weed and crop species showed differential susceptibility to AAL-toxin, it was patented as a herbicide.[31] Results of further investigations into the chemistry, biological activity and mode of action of the toxin are presented here together with a review of some of our recent work and related studies.

2 CHEMISTRY

AAL-toxins are long-chain alkylamines with one tricarboxylic acid moiety attached (Fig. 1); five types of AAL-toxin have now been described, each with two isomers.[17] The type TA, the most active and produced in greatest quantities by *A. alternata*, has a relative molecular mass of 522 and was the form used in these experiments.[32,33]

AAL-toxin is structurally related to the fumonisins

(Fig. 1) which include two tricarboxylic acid moieties.[18,21,26,34] Fumonisin B_1 (FB_1) is well-known as a mammalian toxin[15,21,34,35] and is known to be the cause of equine leukoencaphalomalacia[36] and pulmonary edema in swine.[37–39]

FB_1 has been well studied by us and by others and has been demonstrated to be a non-host-specific phytotoxin to a wide variety of dicotyledonous weed and crop species.[17,19,22,24,26,27,40,41] Its further study as a herbicide has been limited because of possible concerns about carcinogenicity in rats.[42]

The mechanism of action of FB_1 in animals has been shown to be disruption of sphingolipid metabolism.[43–46] Sphingolipids are important constituents of cell membranes in both animals and plants,[34,47–49] but their role in plants is not well studied. Both AAL-toxin and the fumonisins inhibit sphinganine (sphingosine) *N*-acyltransferase (ceramide synthase in animals),[44,45] apparently as a result of structural similarities between the toxins and sphingolipids (Fig. 1), suggesting that these compounds are competitive inhibitors of the enzyme. Cerebrosides, major components of plant plasma membranes, consist of a nitrogenous alcohol, a fatty acid and a monosaccharide moiety.[21,34,47,49] Sphinganine and AAL-toxin have respectively an 18-carbon and a 17-carbon alkylamine backbone, with sphinganine having an *N*-terminal methanolic substituent and AAL-toxin having multiple substituents at the *C*-terminal end. AAL-toxin apparently competes with sphinganine and other sphingolipids for the enzyme sphinganine (sphingosine) *N*-actyl transferase, and causes accumulation of sphinganine and depletion of complex sphingolipids. Therefore, the effect of AAL-toxin can be determined by measuring the build-up of free sphingoid bases in plant and animal systems.

3 BIOLOGICAL ACTIVITY

AAL-toxin causes phototoxic damage on susceptible tomatoes including chlorosis, necrosis, stunting, leaf curl, wilt, and mortality at concentrations of $\leqslant 5$ μM when sprayed onto plants. Symptoms appear 24 to 48 h after exposure.

The toxin is also phytotoxic to a variety of other plant species.[28–30] At the low concentration of 5 μM, phytotoxic effects of varying degrees are seen in the following families: Leguminosae on hemp sesbania (*Sesbania exaltata* Rydb., ex A.W. Hill); northern jointvetch (*Aeschynomene virginica* (L.) B.S.P. and soybean (*Glycine max* (L.) Merr.); Lemnaceae on duckweed (*Lemna pausicostata* L.) and common duckweed (*L. minor* L.); Malvaceae on prickly sida (*Sida spinosa* L.), spurred anoda and venice mallow (*Hibiscus trionum* L.) and Solanaceae on susceptible tomatoes, jimsonweed and black nightshade (*Solanum nigrum* L.). At higher concentrations (1 mM), many other plants show some susceptibility. Heterozygous (*Asc/asc*) and resistant tomatoes showed minimal effects of 1 mM concentration that did not progress with time. Monocotyledonous plants are largely resistant.

Duckweed is a small aquatic plant that is sensitive to AAL-toxin and the fumonisins. It serves as a target plant for bioassay of AAL-toxin and is easily used as an intact plant to determine electrolyte leakage and chlorophyll loss, which indicate cell membrane disruption.[50] The lowest concentration of the toxin that causes significant electrolyte leakage and chlorophyll loss in duckweed is 20–50 nM. Significant leakage is not measurable for 48 h and leakage increases over 72 h. FB_1 in the same bioassay system is 10-fold less effective.[26]

We also tested various sphingoid bases for phytotoxicity in the duckweed bioassay system as measured by electrolyte leakage and chlorophyll loss. At concentrations of 10 to 100 μM (*c.* 100-fold greater than AAL-toxin) phytosphingosine and sphingosine caused symptoms similar to those caused by AAL-toxin and FB_1[26] (Figs 2 and 3).

In the tomato leaf disc bioassay system, at 1 μM, both AAL-toxin and FB_1 caused electrolyte leakage and chlorophyll loss.[6] The more complex structure and the thickness of the tomato leaf may account for the

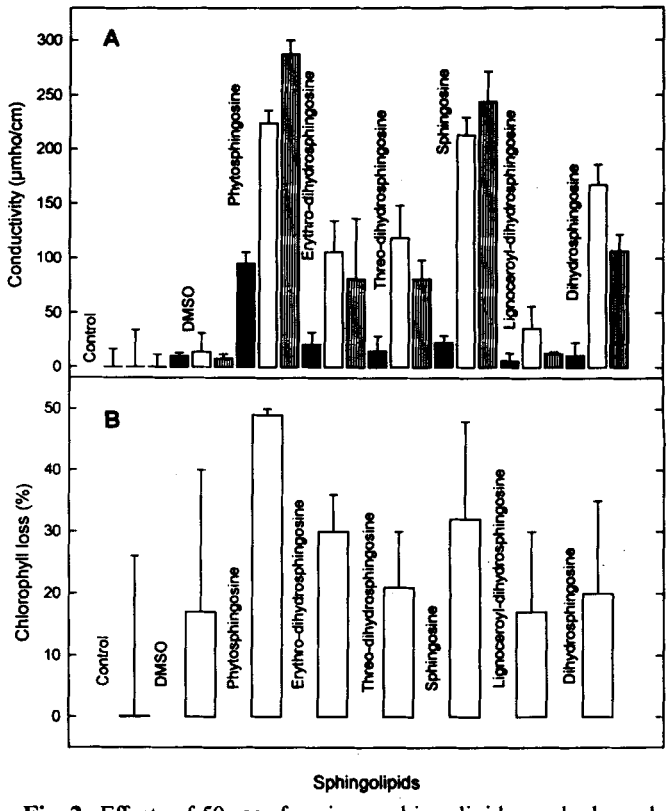


Fig. 2. Effects of 50 μM of various sphingolipids on duckweed. (A) Cellular leakage, as determined by change in electrical conductivity with respect to the control of the bathing media (■) 24, (□) 48, and (▥) 72 h after exposure to each compound, and (B) chlorophyll loss after 72 h of exposure. Error bars are 1 S.E. of the mean. (Modified from Ref. 26).

TABLE 1
Response of Leaf Discs Obtained from One-Month-Old Resistant and Susceptible Tomato Lines Grown under Greenhouse and Growth Chamber Conditions to AAL-toxin and FB_1[a]

Variety and (genotype)	*Toxin*	*Rate (μM)*	*Growth condition*	*Cellular toxicity at 48 h*	
				Electrolyte leakage increase (%) (±S.E.)	*Chlorophyll loss*
Ace	AAL-toxin	100	Greenhouse	93 (±5·2)	10 (±3·4)
(Asc/Asc)	FB_1	100	Greenhouse	139 (±6·2)	1 (±0·2)
	AAL-toxin	100	Growth chamber	107 (±7·7)	13 (±5·5)
	FB_1	100	Growth chamber	115 (±17·6)	1 (±0·6)
LA12	AAL-toxin	1	Greenhouse	112 (±8·7)	1 (±0·9)
(asc/asc)	FB_1	1	Greenhouse	310 (±20·5)	25 (±12·8)
	AAL-toxin	1	Growth chamber	476 (±15·6)	22 (±8·4)
	FB_1	1	Growth chamber	163 (±9·5)	13 (±3·6)
#85-6	AAL-toxin	1	Greenhouse	479 (±15·6)	36 (±7·4)
(asc/asc)	FB_1	1	Greenhouse	276 (±10·0)	29 (±7·3)
	AAL-toxin	1	Growth chamber	192 (±5·7)	4 (±2·4)
	FB_1	1	Growth chamber	115 (±6·6)	11 (±3·5)

[a] Results are with respect to control. All experiments are the mean of three replicates repeated twice.

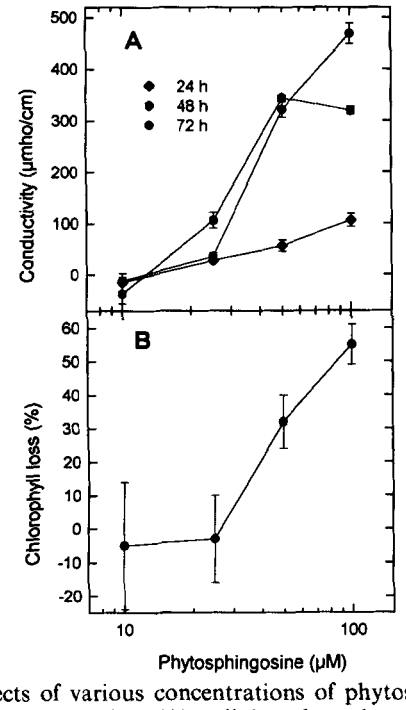


Fig. 3. Effects of various concentrations of phytosphingosine on duckweed, including (A) cellular electrolyte leakage as determined by change in electrical conductivity with respect to the control of the bathing media (◆) 24, (◆) 48, and (●) 72 h after exposure to the compound, and (B) chlorophyll loss after 72 h of exposure. Error bars are 1 S.E. of the mean. In some cases, the datum symbol is larger than the standard error bar. (Modified from Ref. 26.)

somewhat higher concentrations that are required than in duckweed.

In leaf disc bioassays with one-month-old tomato plants, the relative phytotoxicities of AAL-toxin and FB_1 varied according to whether the discs were obtained from plants grown in greenhouse or in a growth chamber. Greenhouse growth conditions were 28–33°C, 40–60% RH and a photoperiod of 1600–1800 $\mu E\ m^{-2}\ s^{-1}$ at midday for 1 year, and those for the growth chamber were 28–30°C, 80 (±5)% RH and a photoperiod of 300 $\mu E\ m^{-2}\ s^{-1}$ for 14 h. Three lines of tomatoes, the resistant line 'Ace' with genotype (*Asc/Asc*), the susceptible line 'LA12' with genotype (*asc/asc*), and the susceptible line 85–6 with genotype (*asc/asc*) were used with the same technique described in detail by Abbas *et al.*[6,30] Electrolyte leakage and chlorophyll loss were measured (Table 1). The experiment was repeated twice with three replicates each.

FB_1 seemed to be more active against susceptible tomatoes relative to AAL-toxin when plants were grown in in the greenhouse rather than in the growth chamber. However, results were not uniform among the two susceptible varieties.

Both tomato leaf discs and duckweed are useful bioassays for determining stability of the toxins. AAL-toxin (100 μM) in aqueous solution was stored under refrigeration (1–4°C) for over one year. Leaf discs from susceptible tomatoes grown under the same conditions (greenhouse and growth chamber) showed identical phytotoxicity with freshly prepared and stored solutions of the toxin. Duckweed also showed the same susceptibility to the old and new solutions.

4 ULTRASTRUCTURAL EFFCTS

As data were available for sphingolipid accumulation in susceptible tomato leaf discs, an attempt was made to

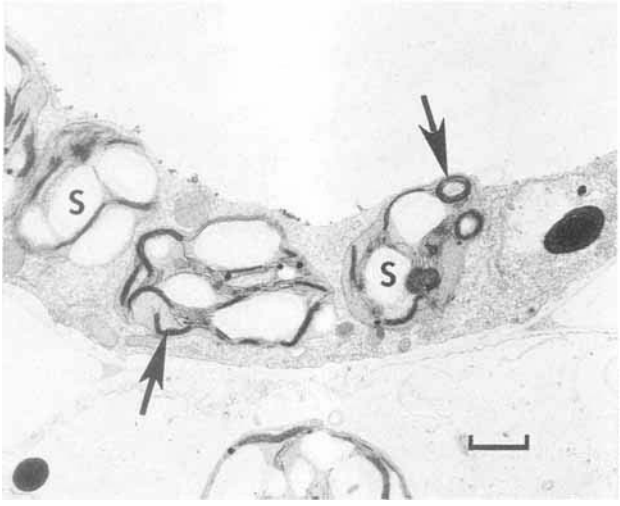


Fig. 4. Ultrastructure of mesophyll cell of black nightshade treated with AAL-toxin (25 μM) 30 h previous to fixation. Arrows show unusual grana conformations induced by the toxins; S = starch grain. Bar = 1μM.

correlate changes in sphingolipid metabolism with ultrastructural effects. However, the tomato leaf discs proved to be difficult materials for electron microscopy.

Nonetheless, ultrastructural studies were conducted on black nightshade leaf discs exposed to AAL-toxin at 25 μM; black nightshade shows a similar susceptibility as *asc/asc* tomatoes to AAL-toxin. Compared to the control, the results of electron microscopic examination showed very little ultrastructural changes up to 24 h but at 30 h (Fig. 4), cytological damage was seen in the treated tissues. Grana stacks exhibited morphological abnormalities such as circles or cups (Fig. 4, arrows) and some chloroplast envelopes entirely disappeared. Mitochondria and ribosomes were still present. After 36 h, no structures other than cell walls, starch grains and still-stacked grana were identifiable. A control plant at 36 h was normal, other than distorted chloroplasts secondary to starch buildup. After 48 h, treated plants had no intact organelles and only membrane debris was seen.

We plan to continue this investigation by studying sphingolipid accumulation in black nightshade in order to correlate all measurements (including sphingolipids, electrolyte leakage, decreased chlorophyll content, and ultrastructural effects) on one plant species.

5 MODE OF ACTION

Information quoted in this section is the unpublished work of Abbas *et al.* unless stated otherwise. Exposure to AAL-toxin or fumonisin B_1 causes large increases in the free phytosphingosine and free sphinganine concentration in duckweed, tomato leaf discs, intact tomato plants, or tobacco callus. The increases in free sphingoid bases are easily detected by HPLC of the base-hydrolyzed lipid extracts[51] (Fig. 5). In duckweed and leaf discs from

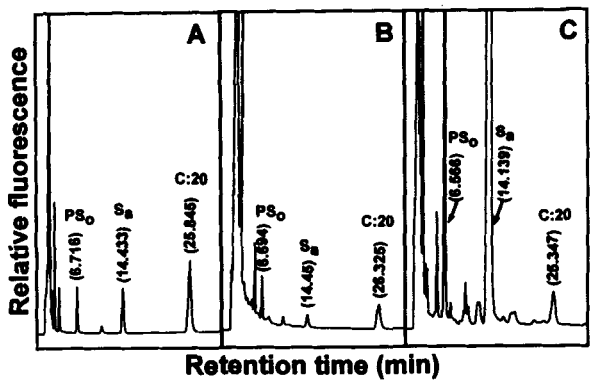


Fig. 5. HPLC profile of free phytosphingosine (P_{SO}), free sphinganine (S_A), and C20 sphinganine for (A) a standard solution, and extracts from susceptible tomato leaf discs exposed for 24 h to (B) distilled water, or (C) 1 μM AAL-toxin.

susceptible tomato varieties, significant increases in free phytosphingosine and free sphinganine are observed after 24 h with 0·1 μM AAL-toxin and at 1 μM, significant increases in free phytosphingosine and free sphinganine occur as early as 0·5 h and 2 h, respectively. Significant increases in free phytosphingosine and free sphinganine occur long before increased electrolyte leakage (compared to controls) in both duckweed and tomato leaf discs.

Responses similar to those seen in duckweed and tomato leaf discs are observed in intact tomato plants exposed to pure AAL-toxin ($\geq$95% purity) or fumonisin B_1 ($\geq$93% purity). Susceptible varieties of tomatoes (*asc/asc*) exhibit significantly larger increases in free sphingoid bases compared to resistant varieties (*Asc/Asc*). The results obtained thus far indicate that AAL-toxin- or fumonisin-induced disruption of sphingolipid metabolism is an early event in the cascade of events leading to cell death. Although the exact enzymatic target for AAL-toxin and fumonisin in plants has yet to be determined precisely, the large increase in free sphinganine suggests that the target in plants may be quite similar to that in animals (Fig. 6); i.e. sphinganine (sphingosine) *N*-acyltransferase.[45,46]

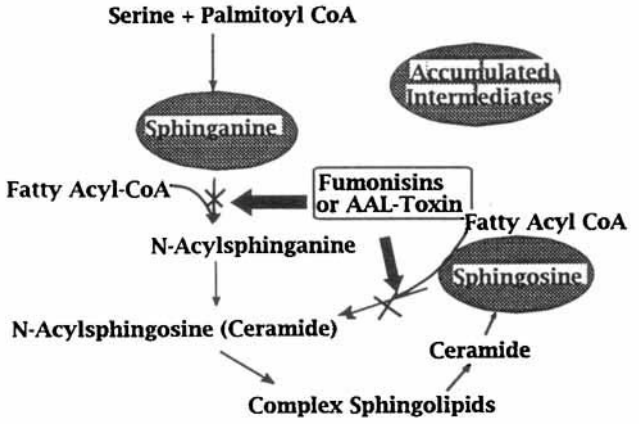


Fig. 6. Scheme showing sphingolipid pathways in animals and site of AAL-toxin/fumonisin inhibition. (Modified from Refs 21 and 45).

Ultrastructural effects of the toxin on black nightshade also indicate that membranes are important targets for AAL-toxin and fumonisins. The earliest effects are seen on the membranes of organelles, while interior structures remain intact for longer periods of time. Electrolyte leakage is also known to result from membrane disruption and occurs early in the course of treatment with AAL-toxin.

6 CONCLUSIONS

AAL-toxin, a natural product, has a wide range of phytotoxicity. It has potential as a natural herbicide because several important weeds including jimsonweed, black nightshade, prickly sida and hemp sesbania are quite sensitive, while some crops such as cotton and maize are not affected. This differential susceptibility may allow its exploitation for weed control.

The data obtained to date are consistent with the hypothesis that the toxin disrupts sphingolipid metabolism and it is hoped that future studies will confirm these preliminary findings.

More research is needed in the field to determine feasibility of commercial development of the toxin and its analogues as natural herbicides. Attention to human and mammalian toxicity will also be required.

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